

Preferential repair of the transcribed DNA strand in plants

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UV-induced pyrimidine dimers block the progression of both DNA and RNA polymerases. In order to reduce the disruptive effect of these lesions on gene expression, bacteria, yeasts, and animals preferentially repair the transcribed strand of actively expressed genes, essentially employing the stalled polymerase as a detector for bulky lesions. It has been assumed, but not demonstrated, that this prioritization of repair also occurs in plants. Here we demonstrate that in the constitutively expressed gene encoding the RNA polymerase II large subunit cyclobutane pyrimidine dimers are removed from the transcribed strand more rapidly than from the non-transcribed strand.

Keywords: transcription-coupled repair, UV repair, pyrimidine dimers, nucleotide excision repair

INTRODUCTION

RNA polymerases, like most DNA polymerases, stall at many DNA damage products. This stalling disrupts transcription of the gene until repair can be effected. By sequestering the transcriptional apparatus, DNA damage can also shut down transcription of undamaged genes. For this reason, the repair of transcriptionally active DNA or at least of the transcribed strand would be expected to be a priority for the cell. Transcription-coupled repair (TCR) was first discovered by Hanawalt and coworkers (Bohr et al., 1985; Mellon et al., 1987). Working with the *DHFR* gene in mammalian cells, they found that pyrimidine dimers in transcriptionally active DNA were repaired more rapidly than those in neighboring non-transcribed sequences, and that this enhanced repair was entirely due to the elevated rate of repair of the transcribed strand. They proposed a general model that still stands today (Sarasin and Stary, 2007): that the presence of a stalled polymerase facilitates the recruitment of nucleotide excision repair (NER) components. This observation has since been extended to include not only NER of other UV-damaged RNA pol II-transcribed genes, but also NER of Pol I-transcribed genes (Conconi et al., 2002; Iben et al., 2002; Meier et al., 2002), and the repair of oxidative damage (Le Page et al., 2000; Sunesen et al., 2002; de Waard et al., 2003).

Transcription-coupled repair has been shown to exist in a variety of mammals and in yeast (Lommel et al., 2000). The adaptive significance of this prioritization of repair is illustrated by the presence of an evolutionarily unrelated mechanism in bacteria (Selby and Sancar, 1993). The presence of TCR in bacteria and yeast, with their relatively compact transcriptional units, also indicates that TCR did not evolve simply to help cope with the higher frequency of lesions/gene in mammals, whose genes often include very large

introns. However, although TCR is often regarded as a ubiquitous repair mechanism, its presence or absence has not yet been determined in plants. In this short report we demonstrate that, not unexpectedly, NER of cyclobutane pyrimidine dimers is enhanced in the transcribed strand of the nuclear housekeeping gene encoding the RNA polymerase II large subunit in *Arabidopsis thaliana*.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

Arabidopsis thaliana ecotype Landsberg *erecta* (Ler) was grown on nutritive agar (Kranz and Kirchheim, 1987) as previously described (Fidantsef et al., 2000). Seeds were sown at a density of approximately 1,000 seeds (12.5 mg)/100 mm² plate and then kept at 4°C for 2 days. The plates were then brought to the growth chamber (20°, 16 h day) where they were grown under cool white lamps. The plates were placed in a vertical position to prevent the root tips from growing into the (UV-absorbing) agar.

UV-B IRRADIATION

Five-day-old seedlings were then irradiated with UV-B for a final dose of 0–6 kJ m⁻² using a UV-transilluminator (Fisher Scientific, Pittsburgh, PA, USA) filtered with 0.005 ml cellulose acetate, with a flux rate of 3.53 mW cm⁻² as measured with a UV-B-specific probe (UVP, Inc., San Gabriel, CA, USA). The tops were removed from the plates prior to irradiation. UV-irradiation and harvesting of seedlings was conducted under dim red light (Photolamp 6W, C.P.M., Dallas, TX, USA). We attempt to harvest about 1 g of seedlings for each treatment and/or time point, which was in general obtained by pooling seedlings from three plates. Seedlings from control (unirradiated) plates were harvested at the time of irradiation and frozen in liquid Nitrogen for later DNA extraction. The remaining plates were irradiated and seedlings were either immediately harvested and frozen or kept in the dark for 24 h and then harvested and frozen.

Abbreviations: CPD, cyclobutane pyrimidine dimer; TCR, transcription-coupled repair.

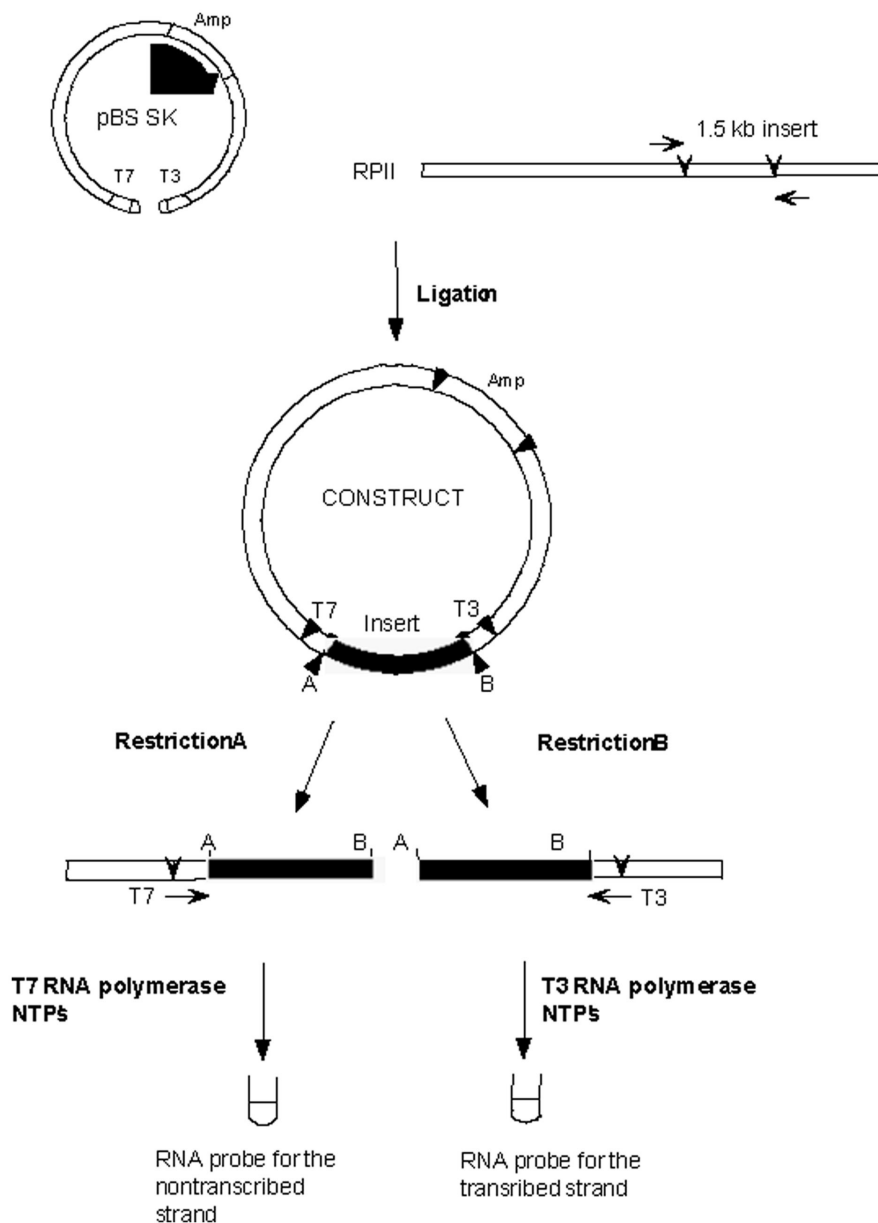


FIGURE 1 | Generation of the single-strand probe for *RPII*. T3 and T7 RNA Polymerase promoters flank the multiple cloning site (MCS) of the pBS SK⁺ vector. The direction of transcription of each RNA polymerase is shown in the dotted arrows. Digestion of pBS SK⁺ with *Bam*HI and *Pst*I linearizes the vector, producing sites for cloning of the 1.5-kb *A. thaliana RPII* insert. The arrow in the insert indicates the direction of transcription of the gene. The 1.5-kb *A. thaliana RPII* insert lies between the T3 and T7 RNA Polymerase promoters of pBS SK⁺. Digestion of the

vector with *Eco*RI linearizes the vector such that the T3 RNA Polymerase makes a transcript complementary to the transcribed strand of the *RPII* gene. Digestion of the vector with *Xba*I linearizes the vector so that the T7 RNA Polymerase makes a transcript complementary to the non-transcribed strand of the *RPII* gene. Addition of RNA synthesis reaction components (appropriate RNA Polymerase and radiolabeled NTPs) produces the probes for detection of each of the DNA strands of the *RPII* insert.

DNA EXTRACTION, DIGESTION, ELECTROPHORESIS, BLOTTING, AND HYBRIDIZATION

DNA was extracted using the DNeasy Plant Maxi Kit (QIAGEN, Inc., Valencia, CA, USA). DNA concentration was measured using a DyNA Quanti 200 Fluoremeter (Hoefer Pharmacia, San Francisco, CA, USA) and digested with *Bst*EII (New England Biolabs, Beverly, MA, USA) in the presence of 5 mM spermidine. Digested

DNA was then aliquoted into two equal volumes. To each aliquot NET buffer was added to a final concentration of 10 mM Tris, pH 7.5, 0.1 M NaCl, 10 mM EDTA, and 100 µg/ml of BSA. T4 endonuclease V (generously provided by R. S. Lloyd) was prepared to a concentration of 1.4 µg/ml in 25 mM NaH₂PO₄ (pH 6.8), 100 µg/ml BSA. To induce nicking at CPDs, 1 µl of this stock solution was added per µg of DNA and the DNA incubated at

37°C for 30 min. To the aliquot not digested with T4 endo V, the T4 endo V dilution buffer was added as a mock digest. The reaction was stopped by adding 1× loading dye to each sample as described (Friedberg and Hanawalt, 1981). Samples (at least 1 µg of DNA/lane) were electrophoresed on a 0.5% alkaline gel (9.5 cm) in 1 mM EDTA and 30 mM NaOH at 22 V for around 16 h. Southern blotting, hybridization and washes were performed as described (Spivak and Hanawalt Philip, 1995) with the exception that Hybond-N+ or Hybond-XL nylon membranes (Amersham Pharmacia, UK) were used. Membranes were exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA).

QUANTITATION OF BAND INTENSITY

Phosphor Screens were scanned in the Storm 860 (Molecular Dynamics) for the quantitation of bands via ImageQuant software (Molecular Dynamics). Quantitation of CPDs was performed as previously described (Chen et al., 1996).

PROBES

A 1.5-kb fragment of the *A. thaliana* *RPII* gene for RNA polymerase large subunit (At4g35800; Nawrath et al., 1990) was used as a probe. The 1.5-kb PCR fragment was obtained using genomic DNA from *Arabidopsis* ecotype *Ler* as a template and the following

primers: 5'-GGTTTGTATGAGGAGGGAGGC-3' (upper primer) and 111 5'-GATGTGGGTGAGTATGATGGAG-3' (lower primer) which amplified the gene from positions 5990 to 7496. This fragment was subcloned into pBluescript SK+ vector between the *Bam*HI and *Pst*I sites. This vector contains the T3 and T7 RNA polymerase promoters positioned in opposite orientations flanking the multiple cloning site of the vector. Digestion of this construct with *Eco*RI or *Xba*I restriction enzymes linearizes the plasmid. The linear vectors produced by digestion with each of these restriction enzymes were separately gel purified. A radiolabeled specific-strand probe that detects the transcribed DNA strand (TS probe) was produced by using the *Eco*RI linearized vector with the T3 RNA polymerase using the Riboprobe *in vitro* Transcription Systems (Promega) following manufacturer's instructions. Similarly, a specific-strand probe that detects the non-transcribed DNA strand (NTS probe) was produced and ³²P labeled by using the *Xba*I linearized vector with the T7 RNA polymerase (see Figure 1).

RESULTS AND DISCUSSION

The Southern blot (aka Bohr, or strand-specific) assay for strand-specific repair relies on the quantitation of dimer-free DNA present in a specific restriction fragment population. In order to accurately measure repair an initial average of at least one dimer/fragment must be generated, and this restriction fragment must lie within a transcribed region. Plants generally have relatively small introns, and so small transcribed regions. This may represent an UV-resistant adaptation, but it more likely reflects the very different process required for splice site recognition in plants vs. animals (Brown and Simpson, 1998). The RNA polymerase large subunit gene, a housekeeping gene expressed in all

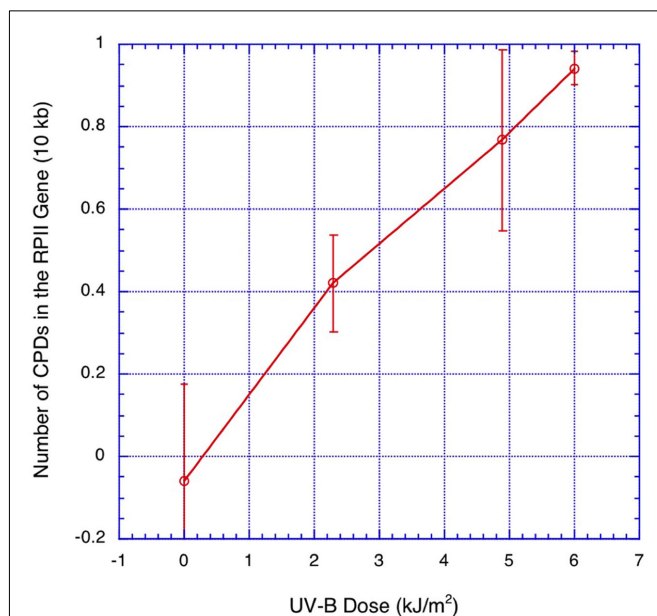


FIGURE 2 | The induction of CPDs as assayed via alkaline Southern

increases linearly with UV-B dose. Five-day-old *Arabidopsis* seedlings grown on 1% agarose plates were exposed to 0, 2.2, 4.8, and 6 kJ/m² of UV-B. Seedlings were immediately harvested in liquid nitrogen after irradiation. DNA samples were digested with *Bst*EII, and divided into two equal aliquots. One aliquot of each sample was subsequently digested with T4 endo V. The digests were run on alkaline gels, and Southern blots were probed with a double strand ³²P labeled probe with a 1.5-kb double-stranded fragment of the *RPII* gene. Signals produced by the probe were measured via phosphorimager. The "0 UV-B" serves as a control for non-specific nuclease activities in the T4 endo V. Values are derived from a minimum of 10 paired (T4 endo+/T4 endo-) lanes representing three biological repeats for each UV dose.

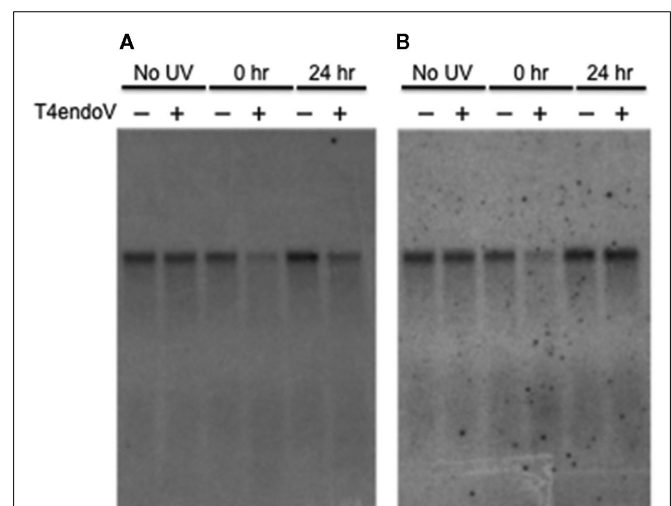


FIGURE 3 | The repair of CPDs in *Arabidopsis* seedlings. Five-day-old *Arabidopsis* seedlings grown on 1% agarose plates were exposed to 0 or 6 kJ/m² UV-B. Seedlings from half of the irradiated plates were immediately harvested and frozen in liquid nitrogen after irradiation (0 h). Remaining plates were stored in the absence of light and seedlings were harvested 24 h after irradiation (24 h). Processing and analysis of the DNA was as described in Figure 2. (A) Blot was probed with RNA probe for non-transcribed strand. (B) The same blot, stripped and reprobed for the transcribed strand.

Table 1 | Preferential repair of CPDs in transcribed strand of the *Arabidopsis thaliana* RPII gene.

	Transcribed strand	Transcribed strand	Non-transcribed strand	Non-transcribed strand
Time (hours)	Dimer frequency	% Dimer removal	Dimer frequency	% Dimer removal
NO UV	0.171 ± 0.052	N/A	0.200 ± 0.056	N/A
0	1.088 ± 0.093	0	1.135 ± 0.101	0
24	0.401 ± 0.174	63	0.942 ± 0.100	17

Values are derived from a minimum of 10 paired (T4 endo+/T4endo−) lanes representing three biological repeats.

tissues, present a large enough (10 kb) target for the Southern blot assay. Irradiation at a UV-B dose sufficient to induce 1 CPD/10 kb is toxic but not lethal to the *Arabidopsis* seedling.

UV-B INDUCTION OF DIMERS IN THE *ARABIDOPSIS* SEEDLING FOLLOWS A POISSON DISTRIBUTION

Calculation of dimer frequency as: $\ln(I^+/I^-)$ (intensity of the band in the +T4 endonuclease V lane/intensity in the paired “no T4endoV” lane) is based on the assumption that dimers are randomly distributed throughout the biological sample in this case, the *Arabidopsis* seedling. We had previously shown that this is true for seedlings using nuclear rDNA (Chen et al., 1994), employing two different methods (the alkaline Sucrose gradient and the Southern blot assay) that produced approximately same results. Here we employed the Southern blot assay on denaturing gels to demonstrate that with increasing doses of UV-B, the induction of dimers in a 10-kb fragment of genomic DNA extracted from *Arabidopsis* seedlings behaved linearly (Figure 2). This indicates that as previously observed UV-B induces a random distribution of dimers in *Arabidopsis* seedlings. This dose–response curve also allowed us to determine the dose required (6 kJ/m² UV-B) to induce an average of approximately 1 CPD/10 kb. This value is consistent with that determined for *Arabidopsis* seedlings in other labs (Draper and Hays, 2000).

DARK REPAIR OF CPDS IS ENHANCED ON THE TRANSCRIBED STRAND

Dark repair of global CPDs in *Arabidopsis* seedlings is very slow in Landsberg *erecta* it is negligible over a 24-h period (Britt et al., 1993; Chen et al., 1996; Tanaka et al., 2002). In order to determine whether CPDs were more efficiently repaired in the transcribed strands of genes, we measured the frequency of CPDs remaining in the transcribed vs. non-transcribed strands of a 10-kb

transcribed fragment of the RNA polymerase large subunit gene. CPD content was measured in seedlings immediately after irradiation and after 24 h. The DNA was restriction digested with *Bst*EII, divided in equal aliquots, one aliquot was treated with T4 endonuclease V, and the resulting preparations loaded onto a denaturing gel. The Southern blot was first hybridized with a probe for the non-transcribed strand of DNA (Figure 3A), the probe was then stripped from the blot, and the blot rehybridized with a probe for the transcribed strand (Figure 3B). Figure 3 shows one representative blot. We measured the signals produced from three independent DNA samples (“biological replicates”). The majority of samples were loaded repeatedly onto the blot, to reduce effects of pipetting error. The CPD values presented in Table 1 represent the average of the values derived for each of the independent samples.

The initial frequency of CPDs was similar in each strand of the irradiated samples (1.1 lesions/10 kb), indicating that both strands have similar susceptibility to DNA damage. DNA from seedlings harvested after a 24-h after UV-B irradiation, however, displayed significantly different frequencies of dimers on the transcribed vs. non-transcribed strands. We found that CPDs persisted in the non-transcribed strand (just as they do in global genomic DNA; Britt et al., 1993; Chen et al., 1994), while being removed specifically from the transcribed strand (Figure 3; Table 1). Our results indicate that the transcribed strand is preferentially repaired over the non-transcribed strand of DNA in this gene, suggesting that the process of TCR is conserved in plants.

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